

Effect of interleukin-1 β and tumor necrosis factor- α on gene expression in human endothelial cells

Baiteng Zhao,^{1,2} Salomon A. Stavchansky,¹ Robert A. Bowden,² and Phillip D. Bowman²

¹Pharmaceutics Division, College of Pharmacy, University of Texas at Austin, Austin 78712; and ²US Army Institute of Surgical Research and Clinical Investigation, Brook Army Medical Center, San Antonio, Texas 78234

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Zhao, Baiteng, Salomon A. Stavchansky, Robert A. Bowden, and Phillip D. Bowman. Effect of interleukin-1 β and tumor necrosis factor- α on gene expression in human endothelial cells. *Am J Physiol Cell Physiol* 284: C1577–C1583, 2003. First published January 29, 2003; 10.1152/ajpcell.00243.2002.—Interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) are two major cytokines that rise to relatively high levels during systemic inflammation, and the endothelial cell (EC) response to these cytokines may explain some of the dysfunction that occurs. To better understand the cytokine-induced responses of EC at the gene expression level, human umbilical vein EC were exposed to IL-1 β or TNF- α for various times and subjected to cDNA microarray analyses to study alterations in their mRNA expression. Of ~4,000 genes on the microarray, expression levels of 33 and 58 genes appeared to be affected by treatment with IL-1 β and TNF- α , respectively; 25 of these genes responded to both treatments. These results suggest that the effects of IL-1 β and TNF- α on EC are redundant and that it may be necessary to suppress both cytokines simultaneously to ameliorate the systemic response.

recombinant cytokines; microarray analysis; human cells; vascular system

ENDOTHELIAL CELLS (EC) lining all blood vessels appear to play an important role during systemic inflammatory responses because of their unique position and immediate exposure to inflammatory mediators. They are known to respond to various stimuli, in part by changing the gene expression for cytokines, adhesion molecules, procoagulation factors, and other proteins. Endothelial dysfunction in systemic inflammation may result in disseminated intravascular coagulation and vascular leakage (12, 16, 23), which may lead to development of multiple organ failure and death. The present information on the response of EC to inflammatory stimuli remains limited. A comprehensive understanding of the response of EC to inflammatory mediators may lead to new means for developing drugs for intervention. The recent modest success in reducing mortality in sepsis with activated protein C, which directly effects the EC and its function in coagulation,

points to its role in the systemic inflammatory response (14).

We previously examined the response of human umbilical vein EC (HUVEC) to gram-negative bacterial lipopolysaccharide (LPS) and found that many genes involving various cellular functions were activated at different times (26). In this study, we explored the response of HUVEC to the proinflammatory mediators interleukin (IL)-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), which are secreted by monocytes and macrophages during systemic inflammatory reactions after infection, inflammation, and tissue damage (18, 25). Their plasma levels correlate significantly with the severity of septic shock and multiple organ failure (2, 3, 11), and they share many biological effects and have been implicated in several acute and chronic pathological states. However, attempts to blunt the systemic inflammatory response by blocking the effects of IL-1 β or TNF- α with receptor agonists or antibodies in severe sepsis have not been successful in reducing overall mortality rate (5, 20). Possible reasons for the ineffectiveness of these trials may include the following: 1) The biological functions of IL-1 β and TNF- α overlap and can complement each other (8). Blocking only one mediator may not effectively reduce the overall inflammatory responses. 2) IL-1 β and TNF- α produce effects at an early stage of inflammation, and the use of their inhibitory reagents at a later stage may not reverse the more damaging events initiated by them. 3) IL-1 β and TNF- α may not represent the best targets for intervention in systemic inflammatory response. Other mediators initiated by them with as yet unknown functions may be better targets.

Therefore, to better understand the EC response to inflammation, we used primary HUVEC with cDNA microarrays containing ~4,000 known human genes to compare the effects of IL-1 β and TNF- α on the alterations of gene expression in EC. About 1% of all genes tested showed significant alterations in mRNA expression levels after stimulation of IL-1 β or TNF- α in EC during a 24-h period. Many of the affected genes appeared in both treatments.

Address for reprint requests and other correspondence: P. D. Bowman, US Army Institute of Surgical Research, 3400 Rawley E. Chambers Ave., Bldg. 3611, Fort Sam Houston, TX 78234-6315 (E-mail: phillip.bowman@amedd.army.mil).

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MATERIALS AND METHODS

EC and treatments. EC from two to four human umbilical veins were harvested by collagenase treatment (133 mg/ml; Roche Molecular Biochemicals, Indianapolis IN), pooled, and seeded in 0.2% gelatin-coated tissue culture flasks in medium 199 containing EC growth supplement (50 µg/ml; Collaborative Biomedical Products, Bedford, MA), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml; Life Technologies, Gaithersburg, MD), and 20% fetal calf serum (Hyclone). The cells were cultured at 37°C in 95% air-5% CO₂.

Primary cultured HUVEC were seeded 1:1 onto gelatin-coated six-well tissue culture plates and, on confluence, were treated with TNF-α (10 ng/ml; Research Diagnostics, Flanders, NJ) or IL-1β (10 U/ml; Roche Molecular Biochemicals) for 1, 4, 7, 12, or 24 h before harvest of total cellular RNA and supernatants. Control cells were treated with the same medium without stimuli for 12 h. Cells in each treatment condition were incubated in duplicate, and each was analyzed with a microarray; cells treated with IL-1β for 12 and 24 h were incubated singularly.

In a nuclear factor κB (NF-κB) inhibition experiment, cells were pretreated with the NF-κB inhibitory peptide SN50 or the inactive control peptide SN50M (Calbiochem, La Jolla, CA) at 50 µM for 1 h before the addition of a stimulus [LPS (50 ng/ml), IL-1β (10 U/ml), or TNF-α (10 ng/ml)] for another 4 h. The parallel positive controls were subjected to the same medium for 1 h without a pretreatment peptide and then exposed to one of the three stimuli for 4 h. A negative control (without pretreatment or stimulus) was incubated for 5 h. By the end of treatments, cell culture supernatants were collected for measurement of secreted monocyte chemoattractant protein type 1 (MCP-1) and IL-8.

RNA isolation, cDNA production, microarray hybridization, and image acquisition. RNA isolation, cDNA production, microarray hybridization, and image acquisition were carried out as described elsewhere (26). Briefly, total RNA was isolated from cells with TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Then 2 µg of total RNA were reverse transcribed into radiolabeled cDNA with [³³P]dATP (10 mCi/mmol; Amersham/Pharmacia, Arlington Heights, IL). Microarrays containing ~4,000 known human genes (GeneFilters GF211 Release I, Research Genetics) were hybridized with the labeled cDNA according to the manufacturer's protocol. The hybridized microarrays were then exposed to a phosphorimaging screen (Packard Instruments, Meridian, CT). After appropriate exposure, high-resolution images were obtained by scanning phosphorimaging screen with a Cyclone scanner (Packard Instruments). The resulting images were analyzed with Pathways 3.0 software (Research Genetics).

Data analysis. Data analysis was performed as previously described (26). Briefly, the intensity of each clone on the microarray was quantitatively analyzed and normalized. Then a statistical analysis, Chen's test (4), was used to determine whether the expression ratio (ratio of treated to unexposed cells) of any gene deviated outside the 99.9% confidence interval for chance-observed magnitudes. The limits of this interval were taken as the screening threshold to identify genes with likely-altered expression.

Expression ratios of these presumably up- or downregulated genes were also used in clustering analysis with Cluster and Treeview programs (Michael Eisen, Stanford University, genome-WWW.stanford.edu). These programs allow genes with similar expression patterns to be grouped to-

gether and displayed in colors representing induction and suppression.

Real-time RT-PCR. Real-time PCRs were performed with 1 µg of total RNA from the same samples used for microarray using a LightCycler thermal cycler (Idaho Technology, Salt Lake City, UT) as previously described (26). Briefly, total RNA was reverse transcribed to cDNA with Superscript II reverse transcriptase and poly(dT) priming (Life Technologies), and 1 µl of cDNA solution was amplified with a primer set for each gene listed below. The amplification reaction was stopped at the exponential range, and all the resultant PCR products were displayed with gel electrophoresis on 2% agarose containing 1:10,000 SYBR gold nucleic acid stain (Molecular Probes, Portland, OR). The gene-specific primers and the size of the PCR products were as follows: sense and antisense for β-actin (131 bp), CCTCCAGCATGAAAG-TCTCTGC (sense) and AGTGTTCAGTCTTCGGAGTTTGG (antisense) for MCP-1 (313 bp), ATGACTTCCAAGCTGGC-CGTGGCT (sense) and TCTCAGCCCTCTTCAAAAACCTCTC (antisense) for IL-8 (289 bp), CAAACCGAAGTCATAGCCA-CACTC (sense) and TCTCCTAAGCGATGCTCAAACAC (antisense) for GRO1 (251 bp), and acaaatcagacggcagcactg (sense) and GGCACCTCTTTTCATAAGGGG (antisense) for plasminogen activator inhibitor type 1 (PAI-1, 169 bp).

ELISA. Cell culture supernatants were collected at the end of treatment and stored at -20°C until analyzed by ELISA for IL-1β, MCP-1, and IL-8 (R & D Systems, Minneapolis, MN). Samples were assayed according to the manufacturer's instructions. The detection sensitivity is 1 pg/ml for IL-1β and 5 pg/ml for MCP-1 and IL-8 in cell culture media. Data were analyzed with the Tamhane T2 test using SPSS software.

RESULTS

Assessment of the reproducibility of the microarrays. To assess the reproducibility of the results obtained from these cDNA microarrays, duplicate samples labeled and hybridized on two microarrays from the same treatment were compared (Fig. 1A). The normalized intensities of any clone from duplicate samples have a ratio approaching 1. The two parallel lines in Fig. 1 were the screening thresholds as described in *Data analysis*. Only clones falling above or below the lines were considered to have different expression between samples. Five clones (0.1% of all clones) showed expression difference between the same duplicate samples, i.e., the expected false rate. Comparison between a single control replicate and a single 4-h TNF-α-treated sample replicate resulted in more gene expression changes at greater deviations (Fig. 1B). For actual sample screening, microarray duplicate means were plotted on the axes when duplicates were available.

Identification of differentially expressed genes induced by IL-1β. To examine the EC response to IL-1β at the level of gene expression, confluent HUVEC cultured in six-well tissue culture plates were given fresh medium with or without 10 U/ml recombinant human IL-1β and incubated for various times. At the end of treatment, total RNA was isolated and subjected to cDNA microarray analysis. A total of 33 genes with expression ratios beyond screening thresholds at 99.9% confidence limits during 24 h of stimulation were identified as responsive to IL-1β in EC. These genes are

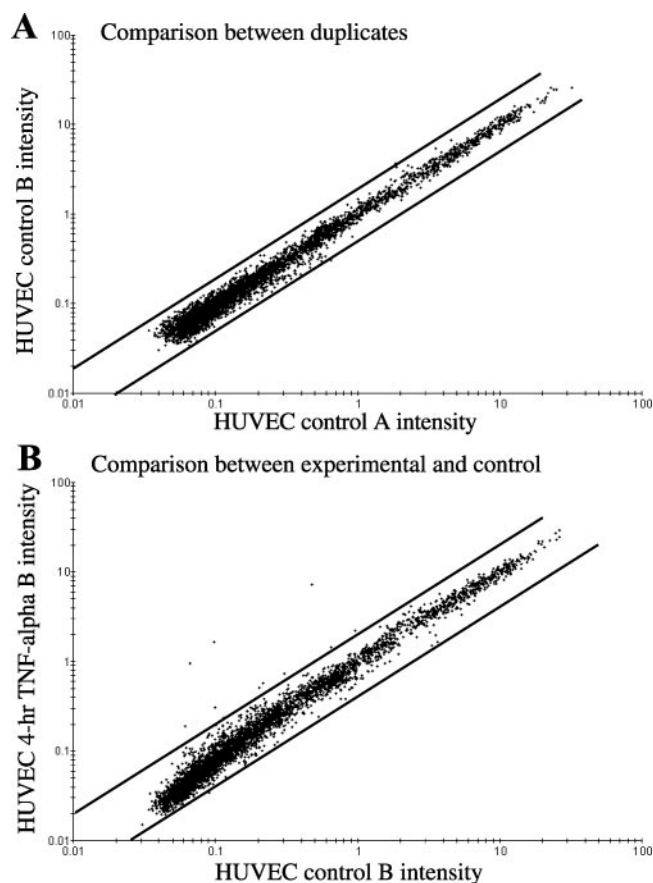


Fig. 1. Correlation of microarray analysis between hybridizations for 2 separate control duplicates (A) and for a control and a sample treated with tumor necrosis factor- α (TNF- α) for 4 h (B). Total cellular RNA from different samples was isolated, reverse-transcribed, and hybridized to different microarrays. Acquired images were analyzed by computer program, and clone intensities from each sample were compared to identify transcriptionally changed genes. A spot outside the 2 parallel lines represents a gene outside the 99.9% confidence limit (screening threshold). HUVEC, human umbilical vein endothelial cells.

clustered in Fig. 2 on the basis of the similarity of their expression patterns with Cluster and Treeview programs.

The number of genes with changed expression levels at each time point is shown in Fig. 3. The mRNA levels of 11 genes (33% of 33) were changed beyond the screening threshold after 1 h of stimulation of IL-1 β , but most of them recovered to control levels within 24 h. The other 22 genes were affected at later times and had different induction or repression patterns. Some of these genes displayed a short temporal expression disturbance; the others apparently remained changed for ≥ 24 h. According to fold changes, the most upregulated genes are the CXC-type chemokine IL-8 and the CC-type chemokine MCP-1, which induce extravasation of neutrophils and mononuclear cells, respectively. These two proinflammatory chemokines were upregulated as early as 1 h and sustained for up to 24 h. Only two genes (PRKAR2B and HMGIY) appeared suppressed in EC by IL-1 β treatment. Expression of some genes known to be involved in the re-

sponse of EC in inflammation provides a measure of confidence of this screening technique.

Gene expression profiling of TNF- α -affected genes. To examine the endothelial response to TNF- α , the same experimental procedure was performed using TNF- α (10 ng/ml) in place of IL-1 β . Microarray analysis identified 58 genes with mRNA expression ratios (ratio of exposed to unexposed samples) that changed beyond screening thresholds in human EC after TNF- α stimulation. They were clustered together with the genes identified from the IL-1 β experiment and displayed in Fig. 2. The number of these identified genes at different times is shown in Fig. 3. At 1 and 4 h, fewer genes were affected by TNF- α than by IL-1 β . Only five genes (9% of 58 genes) were upregulated within 1 h of treatment. Most genes affected by TNF- α were identified at 7 and 24 h. Nine genes (15% of 58 genes) appeared downregulated over a 24-h period.

IL-8 and MCP-1 showed the same expression patterns as in IL-1 β experiment. A section of the images from scanned cDNA microarrays containing the spot for MCP-1 is shown in Fig. 4. Comparison of the results from both experiments shows a common group of 25 genes apparently affected by both cytokines. They account for 75% and 43% of all the identified genes in IL-1 β and TNF- α treatments, respectively. The time pattern of expression of many of these genes was similar with both agents. They belong to different functional groups, such as chemokine (e.g., IL-8 and MCP-1), extracellular matrix related (e.g., PAI-1 and matrix metalloproteinase type 10), inflammation (e.g., NK4, HLA-C, and B2M), signal transduction (e.g., ARHB, TRAF1, PRKAR2B, and CAV1), and metabolism (e.g., SAT, EXT1, NNMT, and PTGS2). Thirty-three genes affected only by TNF- α and eight by IL-1 β in EC. Some of the TNF- α -specific genes were ADD3, SCYA14, ALDH1, PRG1, PSME1, PSME2, PSMD9, GARS, SARS, SUI1, TSFM, TNFRSF11B, JUN, and TGFBI1. The IL-1 β -specific genes included HMGIY, GRO1, ZFP36, PAI2, CD47, KDR, PLCB3, and FOSL1. The changes in expression were independently verified with real-time PCRs and gel electrophoresis for some of these genes (Fig. 5). In general, microarray and PCR methods gave similar results.

To determine whether TNF- α could induce IL-1 β production in HUVEC, cell culture supernatants from the TNF- α experiment were subjected to ELISA. IL-1 β was not detectable, indicating that the response of EC to TNF- α was not likely the result of induction of IL-1 β (data not shown).

Effect of NF- κ B inhibition on MCP-1 and IL-8 secretion induced by IL-1 β and TNF- α . In an earlier study, it was shown that induction of MCP-1 and IL-8 by LPS is exclusively through the NF- κ B pathway (26). To determine whether they are also NF- κ B dependent, the NF- κ B inhibitory peptide SN50 and the inactive control peptide SN50M were given to the cells before the cytokine treatments. LPS was used as a positive control on the effectiveness of the peptides. SN50 did not inhibit IL-1 β - or TNF- α -induced MCP-1 and IL-8 secretion by blocking the translocation of NF- κ B but was

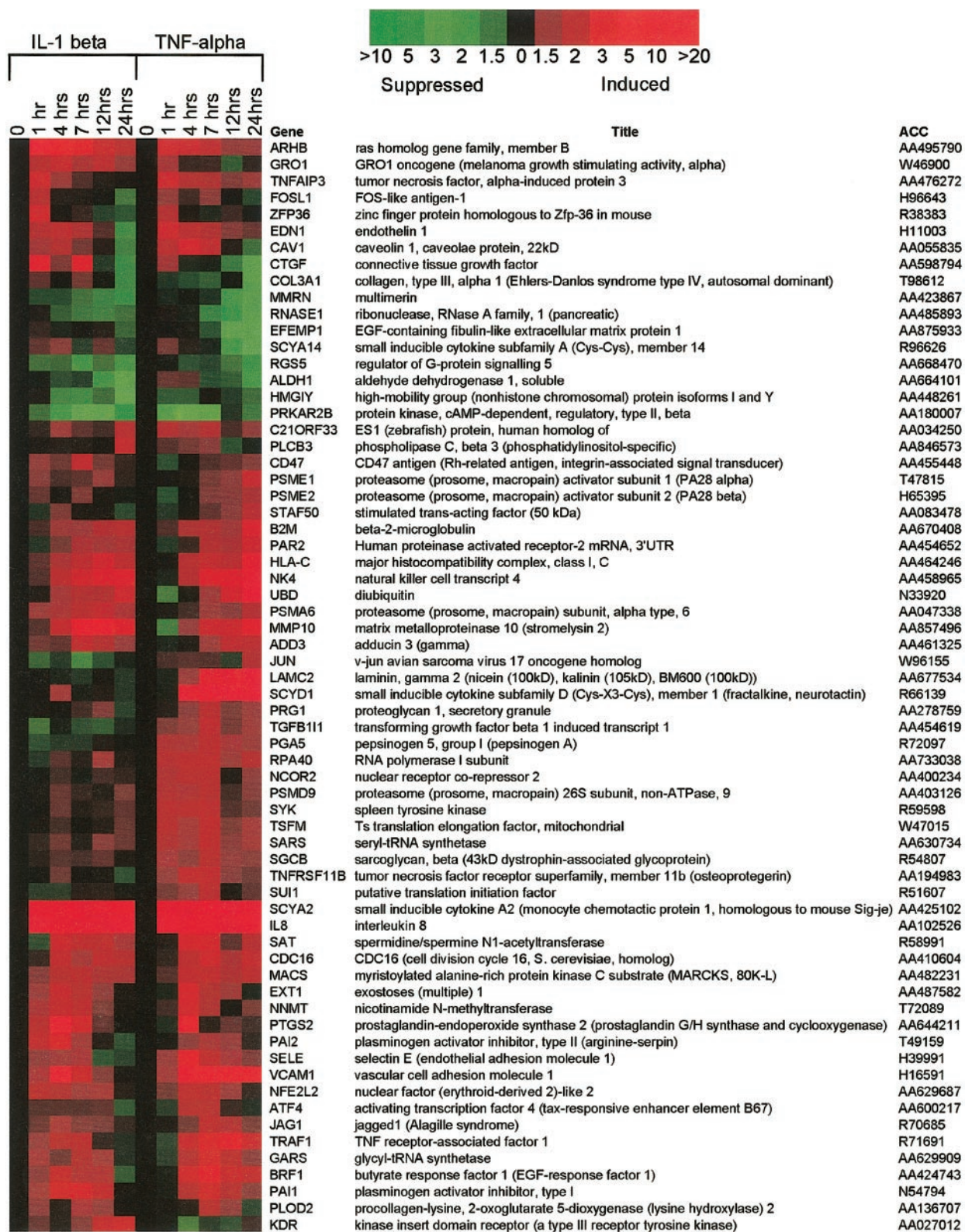


Fig. 2. Cluster analysis of expression profile of interleukin (IL)-1 β - and TNF- α -treated HUVEC. Cluster analysis was performed with Cluster and Treeview programs. Ratios of each gene relative to controls at different times after IL-1 β or TNF- α treatment were used to rearrange the gene list on the basis of their expression pattern. Genes with similar upregulation (red) or downregulation (green) trend were placed close to each other. Magnitudes of ratios are reflected from their color intensity comparable to the color-ratio bar. All genes (total 66) identified in both treatments are displayed, so not every gene passed the screening threshold in both treatment columns. Thirty-three and 58 genes appeared to be affected by IL-1 β or TNF- α , respectively; 25 of these were common in both treatments. EGF, epidermal growth factor.

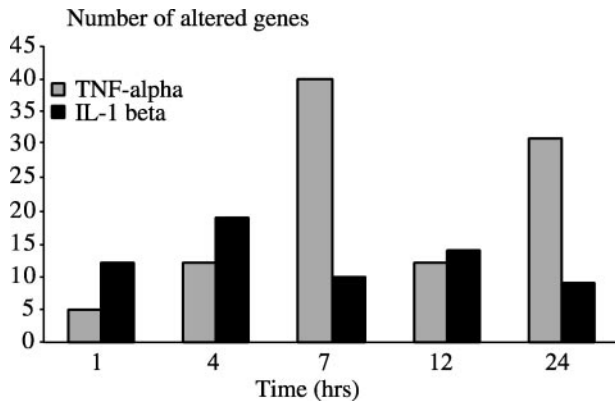


Fig. 3. Number of genes with mRNA levels that changed at different times after cytokine treatment. Number of genes outside screening thresholds at each time point is compared for IL-1 β and TNF- α treatment. TNF- α initially affected fewer genes but induced more gene expression changes than IL-1 β at 7 and 24 h. Some genes were altered in all time points; others had a temporal expression change.

effective in blocking LPS stimulation of their production (Fig. 6).

DISCUSSION

During inflammation, presumably protective responses may be inappropriately overexpressed, potentially resulting in morbidity and sometimes mortality. The proinflammatory cytokines TNF- α and IL-1 β are potent cytokines with dramatic effects on many cells. By utilizing cDNA microarray techniques that allow parallel, high-throughput screening of altered gene expression, the identification of cytokine-affected genes becomes possible. The present study investigated the effects of TNF- α and IL-1 β on regulation of gene expression in EC among ~4,000 genes.

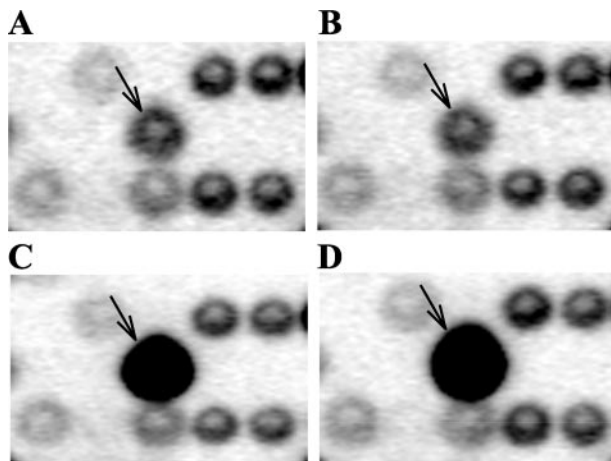


Fig. 4. Upregulation of monocyte chemoattractant protein type 1 (MCP-1) mRNA levels in HUVEC in response to TNF- α . Confluent HUVEC in 6-well tissue culture plates were treated with or without TNF- α (10 ng/ml) for various times. mRNA levels of thousands of genes were analyzed by cDNA microarray techniques. Sections of acquired microarray images containing the spot for the MCP-1 gene (arrows) are shown for duplicate controls (A and B) and for samples treated with TNF- α for 4 h (C and D). MCP-1 represents one of the genes most upregulated by cytokines in HUVEC.

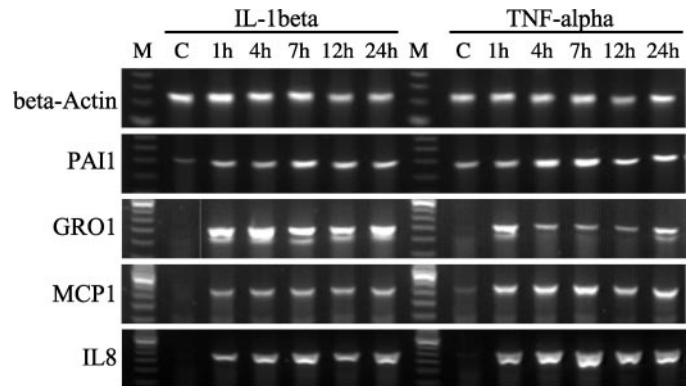


Fig. 5. Independent verification of microarray results with real-time PCR. From each sample, 1 μ g total RNA was reverse-transcribed into cDNA, and indicated genes were amplified with a LightCycler thermal cycler. Resultant PCR products are shown after gel electrophoresis on 2% agarose. M, DNA molecular weight marker; C, control; PAI, plasminogen activator inhibitor.

TNF- α and IL-1 β are the products of genes with little homology and bind to different cell surface receptors. However, activation of their receptors leads to the induction or suppression of a similar set of genes in HUVEC, including genes for chemokines, cell adhesion molecules, procoagulants, metalloproteinases, proteasomes, and the major histocompatibility complex. The receptors for TNF- α and IL-1 β employ similar signaling pathways that involve mitogen-activated protein (MAP) kinase cascades (6, 7, 15). Eder (9) proposed a model in which MAP kinase kinase kinases connect the TNF- α and IL-1 β signaling pathways. A common result of this MAP kinase kinase kinase-primed pathway is the activation of certain transcription factors, such as NF- κ B and activating protein 1 (AP-1), a heterodimer of *c-jun* and *c-fos*. In this study, a significant overlapping of the affected genes by both cytokines was noticed (25 of 33, or 75%, with IL-1 β treatment; 25 of 58, or 43%, with TNF- α treatment). Some of these genes have been well known for their roles in inflammation, such as PAI-1, vascular cell adhesion molecule type 1 (VCAM-1), IL-8, MCP-1, endothelin-1, matrix metalloproteinase type 10, β_2 -microglobulin, TNF receptor-associated factor-1, and prostaglandin-endoperoxide synthase-2. Others, including natural killer cell transcript-4, diubiquitin, butyrate response factor-1, and caveolin-1, have unclear functions with respect to inflammatory responses.

Comparison of the gene expression profile of HUVEC in response to LPS from our earlier study (26) with the results reported here shows that 15 genes are unique to LPS, 30 to TNF- α , and 6 to IL-1 β alone. However, 18 of these 25 genes are stimulated in common (Fig. 7). This finding is supported by the evidence that NF- κ B is also the major transcription factor in LPS-induced gene transcription regulation in HUVEC. Furthermore, the intracellular domain of the transmembrane protein toll-like receptor-4, which is responsible for LPS binding and signal transduction, is very similar to that of IL-1 β (1). Therefore, it is not surprising to find that the group of genes identified in HUVEC in re-

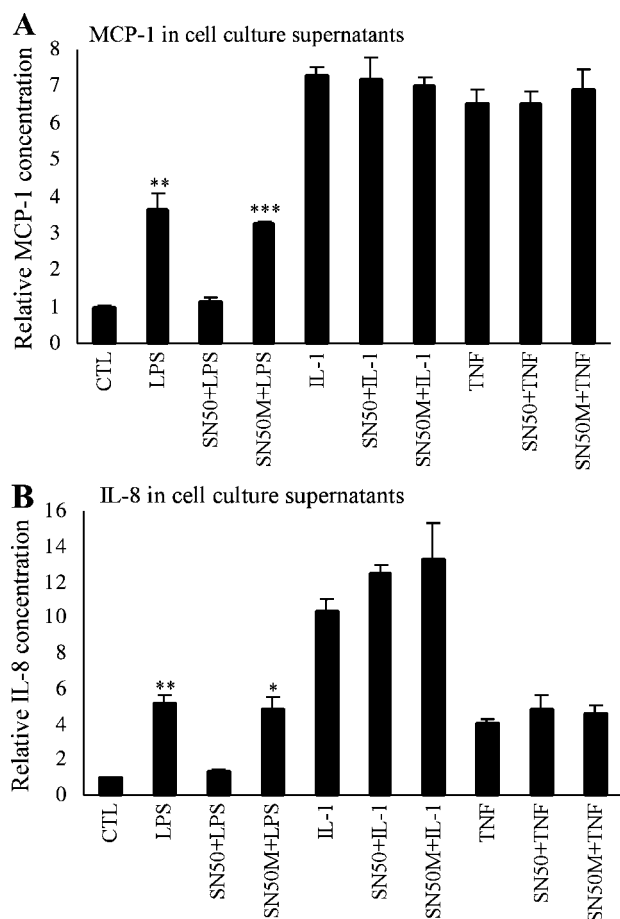


Fig. 6. Differential effect of nuclear factor- κ B (NF- κ B) inhibition on MCP-1 secretion induced by lipopolysaccharide (LPS), IL-1 β , and TNF- α . Confluent HUVEC in 48-well tissue culture plates were pretreated with 50 μ M NF- κ B translocation inhibitory peptide SN50 (SN50 + stimulus) or 50 μ M control peptide SN50M (SN50M + stimulus) for 1 h before administration of *Escherichia coli* LPS (50 ng/ml), IL-1 β (10 U/ml), or TNF- α (10 ng/ml) for 4 h. Negative controls (no stimulus) and positive controls (stimulus only) were done in parallel. Cell culture supernatants were collected at the end of treatments and analyzed by ELISA for MCP-1 (A) and IL-8 (B). Concentrations were normalized to mean of negative controls and shown with standard deviation ($n = 3-5$). Inhibition of NF- κ B translocation greatly reduced stimulated MCP-1 and IL-8 secretion by LPS, but not by IL-1 β or TNF- α . * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with SN50 + LPS.

sponse to IL-1 β and LPS is very redundant. Because IL-1 β was not detectable in culture media from TNF- α - or LPS-stimulated HUVEC with ELISA and TNF- α protein production is not induced by IL-1 β or LPS alone in HUVEC (13, 22), the differential expression patterns induced by these stimuli appeared to be caused by an individual stimulus, not by a combination of two or more stimuli.

Even though TNF- α and IL-1 β exhibited similar patterns of gene expression, they do not share identical signaling pathways and functions. Each of them was able to stimulate a unique set of genes in HUVEC. For example, TNF- α upregulated the expression of several members of the proteasome family, such as PSME1, PSME2, PSMD9, and PSMA6, whereas IL-1 β upregu-

lated only PSMA6. Proteasomes are involved in IL-1 β -induced MCP-1 production in HUVEC (21). The up-regulated proteasome subunits may also imply involvement of an antigen-presenting pathway in the host's defense against pathogen invasion. The kinetics and extent of the altered gene expression by TNF- α and IL-1 β were not exactly the same. VCAM-1 appeared to be stimulated by TNF- α for a longer time than IL-1 β . Suppression of PRKAR2B expression occurred earlier and for a shorter time with TNF- α than with IL-1 β treatment.

The similarity of gene expression regulation by LPS, TNF- α , and IL-1 β does not mean that a gene affected by all three stimuli is always controlled by the same signaling pathway or transcription factor. TNF- α and IL-1 β are able to activate NF- κ B and AP-1 in EC. However, alteration of LPS-induced gene expression is mainly through NF- κ B, as evidenced in our earlier study, in which pretreatment with an NF- κ B translocation inhibitory peptide abolished most of the transcriptional regulation by LPS. In addition, transfection of an LPS receptor, toll-like receptor-4, construct into cells resulted in activation of NF- κ B, but not AP-1 (10). Using MCP-1 and IL-8 as the model genes, we examined whether a gene is mainly regulated by the same transcription factor activated by different stimuli. MCP-1 is one of the genes most affected by LPS, TNF- α , and IL-1 β in HUVEC and has been known to be under the regulation of transcription factors including NF- κ B, AP-1, and sequence-specific transcription factor-1 (17, 24). Inhibition of NF- κ B translocation greatly suppressed LPS-induced MCP-1 and IL-8 secretion but had no effect on their induction by TNF- α and IL-1 β (Fig. 6). This implies that AP-1 and sequence-specific transcription factor-1 may be more potent in some HUVEC responses to these two cytokines.

Results similar to these have been reported for 4 h of TNF- α stimulation of HUVEC by Murakami et al. (19) utilizing Affymetrix chips interrogating 35,000 genes. They reported some of the most upregulated genes, and

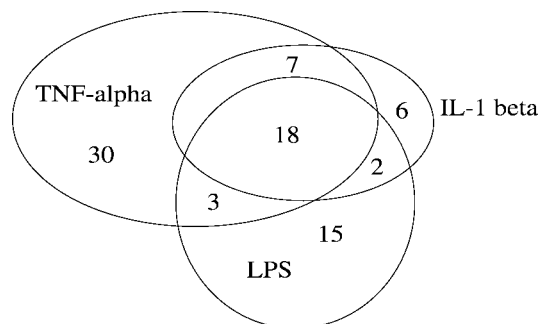


Fig. 7. Number of genes identified in HUVEC in response to LPS, TNF- α , and IL-1 β and their distribution. Each of the 3 ellipses has an area representing total number of genes affected by 1 stimulus. Twenty-five genes are enclosed by IL-1 β (75% of 33 genes) and TNF- α (43% of 58 genes) treatments. By inclusion of earlier observations (26), 23 of the 38 genes responding to LPS also responded to treatment with either cytokine. There may be considerable redundancy in the influence of inflammatory mediators on HUVEC gene expression.

many of them were also found in this study, including TNF receptor-associated factor-1, IL-8, MCP-1, fractalkine, E-selectin, VCAM-1, GRO, and spermidine/spermine N^1 -acetyltransferase.

Taken together, the highly redundant transcriptional effects by proinflammatory agents may point to a partial explanation for the failure of clinical trials attempting to block any single cytokine or LPS in patients succumbing to sepsis and systemic inflammation. The effects of removing one syndrome-causing agent may be compensated by others with similar functions. Thus an agent that interferes with a transcription factor or a step in an involved signaling pathway might prove more efficacious.

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